Exhibit H

#41

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Gordon et al. Examiner: J. Chambers

Serial No: 07/938,322 Group Art Unit: 1804

Filed: August 31, 1992 Attorney Docket: IGI-017

Title: TRANSGENIC ANIMALS SECRETING DESIRED PROTEINS INTO MILK

VIA HAND DELIVERY

Honorable Commissioner of
Patent and Trademarks
Washington, DC 20231

SECOND DECLARATION UNDER 37 CFR 1.132 OF KATHERINE GORDON

1. I hold a Ph.D. degree in Biology from Wesleyan University, and I have worked in the field of molecular biology and gene expression for approximately fifteen (15) years. Currently, I am President and C.E.O. of Apollo Genetics, a biotechnology firm involved working in the field of aging. I was previously employed by Integrated Genetics, Inc. of Framingham, Massachusetts from 1984 to 1989, and then with Genzyme, Inc. from 1989 to 1991 after that company acquired Integrated Genetics, the last position being Associate Director. From the beginning its existence

in 1985, I was responsible for the scientific aspects of the transgenic program at Integrated Genetics, and then at Genzyme after the acquisition of Integrated Genetics.

2. I am the co-inventor of the technology claimed in the above-referenced patent application (attached hereto as Appendix A), and I have carefully studied the patent application. This application discloses methods and gene constructs for producing a recombinant protein which is secreted into the milk of a lactating transgenic animal.

3. I have carefully studied those portions of the U.S. Patent Office Actions dated April 19, 1993 and April 7, 1992, which detail the rejection of the pending claims under 35 U.S.C. §112, first paragraph (at pages 2-4 of the April 19, 1993 Office Action, and pages 2-4 of the April 7, 1992 Office Action, which are attached hereto as Appendices B and C, respectively). I understand that the Examiner has objected to the specification as failing to provide an adequate description of, and enablement for, a method for the production of a recombinant protein in the milk of a transgenic mammal. I also understand that the Examiner has held the specification as not enabling for DNA sequences other than those comprising a whey acid protein promoter, arguing that there is insufficient evidence in the specification to indicate that all milk protein promoters can be used with success for the expression of heterologous polypeptide in a transgenic mammal without undue experimentation. I respectfully disagree. Furthermore, it is my understanding that a declaration showing the generation of a transgenic animal expressing a recombinant protein in its milk using a method as claimed in the present application was requested during the personal interview of 16 June 1993 by the Examiner.

4. Employing a method as claimed in the present patent application, transgenic animals were generated which expressed a recombinant protein in their mammary epithelial glands under lactating conditions, and secreted the recombinant protein into their milk. A DNA construct was generated, as described in the present patent application, comprising the gene for the recombinant

protein placed such that a transcriptional promoter sequence from a milk serum protein gene controlled expression of the recombinant protein. The procedures described below are supported by the above-referenced patent application and could be successfully carried out by one of ordinary skill in the art without any additional inventive contribution over that originally disclosed in the present application.

5. In particular, as described in the present application at pages 7-11, and in the research paper entitled "Production of Human Tissue Plasminogen Activator in Transgenic Mouse Milk" in Bio/Technology (1987) 5:1183 of which I am first author (attached hereto as Appendix D), the promoter and upstream regulatory sequences from the murine whey acid protein (WAP) gene were fused to cDNA encoding human tissue plasminogen activator (tPA) with its endogenous secretion signal sequence. This hybrid gene was injected into mouse embryos, resultant transgenic mice were mated, and milk obtained from lactating females was shown to contain biologically active tPA. In similar fashion, the research paper entitled "Transgenic Production of a Variant of Human Tissue-Type Plasminogen Activator in Goat Milk: Generation of Transgenic Goats and Analysis of Expression" in *Bio/Technology* (1991) 9:835 of which I am a co-author (attached hereto as Appendix E) further illustrates that the claimed method can also be used to generated transgenic goats that expressed a variant of human tPA in their milk using no more than routine experimentation by one of ordinary skill in the art.

6. The tPA gene utilized was a cDNA clone from a human uterus cDNA library. The tPA DNA sequence was determined previously and used to construct the plasmid pt-PAVPI-LP(K) which contained the tPA gene (including the tPA signal sequence) and an SV40 polyadenylation site. See page 10, lines 1-6, and Figure 1 of the present application. The construct, illustrated in Figure A below and Figure 3 of the present application, is a tripartite fusion consisting of 2.6 Kb of upstream DNA from the WAP gene through the endogenous CAP site, tPA cDNA beginning in the untranslated 5' region, and the polyadenylation/termination signals from SV40. Briefly, a

HindIII site was added at the 5' end of the 2.6 Kb WAP promoter sequence by digesting the single EcoRI site in the WAP promoter, filling in the overhang with Klenow and dNTPs, and ligating to it a HindIII linker, all by standard protocols. See page 9, lines 13-25 of the present application; Hennighausen et al. (1982) *Eur. J. Biochem.* 125:131; and Campbell et al. (1984) *Nucleic Acid Res.* 12:8685. The tPA cDNA-SV40 termination/polyadenylation cassette was inserted into the polylinker region of the WAP promoter vector as a KpnI-BamHI fragment. See page 10, line 7 -page 11, line 4 of the present application. I note that the resulting construct was used to transform a derivative of the *E. coli* strain MC1061, and the transformants deposited in the American Type Culture Collection and given ATCC Accession No. 67032.

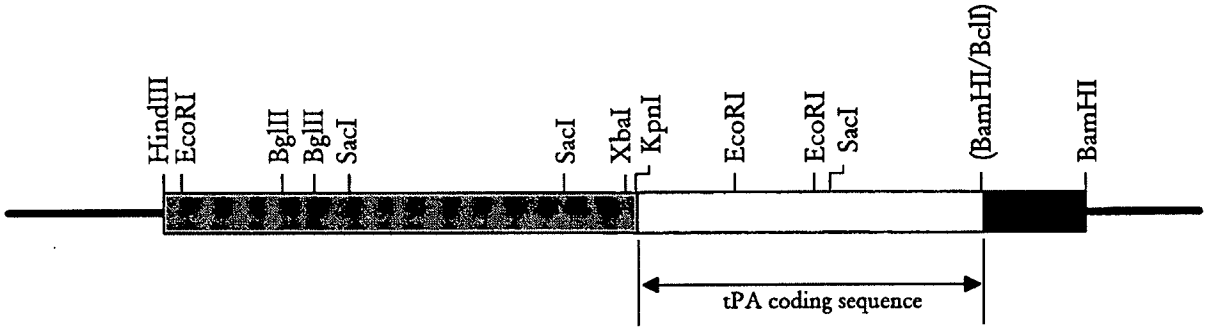

Figure A: Restriction map of WAP-tPA construct. ▣ designates the WAP promoter sequence, □ designates the tPA coding sequence, and ■ designates the SV40 termination/polyadenylation sequence.

A structural tPA variant was also constructed (designated LAtPA) in which an asparagine to glutamine point mutation was introduced into the cDNA of tPA to produce a recombinant protein devoid of glycosylation at the wild-type residue Asn-117. This longer acting tPA variant has been shown have an increased systemic half-life. A DNA fragment containing this point mutation in tPA cDNA was substituted for the equivalent fragment in the WAP-tPA construct above to generate the vector WAP-LAtPA.

7. The WAP-tPA construct was injected into one cell pronuclear mouse embryos as a purified HindIII/BamHI fragment containing no prokaryotic sequences. To purify the eukaryotic

sequences for microinjection, WAP-tPA was digested with HindIII and BamHI, the fragments separated by gel electrophoresis, and the 4.9 Kb fragment purified by binding to glass filter paper. After elution and concentration by ethanol precipitation, the DNA was suspended for microinjection in 10 mM Tris, 0.05 mM EDTA, pH 7.5 at a concentration of 0.5 ng/microliter. The Hind III/BamHI fragment was microinjected into one cell fertilized embryos using standard protocols. See, for example, page 7: line 17 - page 9, line 3, and page 11, lines 5-9 of the present application; Gordon et al. (1983) *Methods in Enzymology* 101:411; Overbeek et al. (1985). *Proc. Nat'l Acad. Sci* 82: 7815-7819; and Gordon et al. (1980) *Proc. Nat'l Acad Sci* 77: 7380-7384. The injected embryos were then implanted into pseudo-pregnant females and allowed to undergo the remainder of gestation.

8. At four weeks of age (post-natal), tail sections were taken from the mice and digested with Proteinase K. DNA from the samples was phenol-chloroform extracted, then digested with various restriction enzymes. The DNA digests were electrophoresed on a Tris-borate gel, blotted on nitrocellulose, and hybridized with a probe consisting of the entire coding region of the tPA cDNA which had been labeled by extension of random hexamers to a specific activity of 1×10^9 cpm/μg. Under conditions of high stringency, this probe did not hybridize with the endogenous mouse tPA gene, and allowed the identification of transgenic mice.

9. Female mice identified as transgenic by diagnostic Southern blot hybridization were mated to wild-type males and had no apparent difficulty in conception or maintenance of pregnancy. Several days after parturition, milk was obtained from the females and was assayed for both tPA protein and tPA activity.

10. Fibrin clot lysis bioassays were performed to assess the tPA activity in milk from the transgenic mice. The fibrin clot assay measured the ability of tPA to digest fibrinogen matrices which were laid down in a background of agarose, thrombin and plasminogen within the cells of

a plate. A small hole was bored through the agarose mixture upon hardening and 25 microliters of sample was loaded into each of the holes. As tPA diffused into the agarose, clearing of fibrinogen was evident visually and the amount of clearing was directly proportional to the amount of tPA activity. The positive controls were generated by addition of varying amounts of recombinant tPA to media composed of either 10% negative mouse milk, 10% negative cow milk, or PBS, to produce a set of dilutions. The negative mouse milk used for these controls was pooled from outbred CD-1 mice in different stages of lactation. By comparison with lysis catalyzed by the known amounts of added tPA in the positive controls, the concentration of tPA activity in the milk of several transgenic mice was determined to be in the range of 200 to 400 ng/ml.

11. The level of tPA protein was quantitated in the milk from three transgenic mice by ELISA, using anti-human tPA polyclonal antibody, and the level of tPA determined to be in the range of 114 to 460 ng/ml. Assays were performed with the IMUBIND ELISA kit produced by American Diagnostics, Inc. The assay was a double antibody sandwich in which the primary antibody was a goat antiserum raised against tPA from human uterus, and the second antibody was a peroxidase conjugated anti-tPA IgG. Standard curves were generated in negative mouse milk diluted to a final concentration of 10% with PBS, to which known amounts of human tPA was added. Milk from wild-type mice showed no signal in the ELISA.

12. The WAP-LAtPA construct was injected into one cell pronuclear goat embryos as a purified HindIII/BamHI fragment containing no prokaryotic sequences. Goat embryos, flushed surgically from the oviducts of superovulated dairy goats, were microinjected with a 4.9 kb HindIII-BamHI fragment of the WAP-LAtPA construct at a concentration of 1 μg/ml in 10mm Tris, pH 7.5, 0.1 mm EDTA, and either immediately transferred to the oviducts of recipient females or cultured in Ham's F12 medium containing 10% fetal calf serum in an atmosphere of 5% CO_2 in air at 37°C for 72 hours and subsequently transferred to the uterus of recipient

females. Typically, the cultured goat embryos were blocked at the 8-16 cell stage in this culture system, but remained viable and could produce offspring. Pregnancies were confirmed by the inability of recipient animals to return to natural estrus, as well as by ultrasonic examination on days 45 and 55 of pregnancy.

13. To describe the generation of transgenic goats in more detail, goats used as donor animals were either Alpine or Saanen breeds. The timing of estrus was synchronized in the donors with norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS; 6 mg). Prostaglandin was administered after the first 7-9 days to remove endogenous sources of progesterone. At day 13 following progesterone administration, follicle-stimulating hormone (FSH, Schering Corp., Kenilworth, NJ) was given to goats at a does of 19 mg over three days in twice daily injections. During the anestrus season (after February), the dose of FSH was increased to 24 mg administered similarly over three days in twice daily injections. Twenty-four hours following implant removal, the donor animals were mated several times to fertile males over a two-day period. Recipient animals were synchronized by the same protocols as the donor animals except that a single non-superovulatory injection of pregnant mares serum gonadotropin (PMSG, Sigma, St. Louis, MO) was given on day 13 of progesterone treatment in place of the FSH. From September to January, the recipients received 400 IU PMSG, and from February to April they received 750 IU PMSG. Recipient females were mated to vasectomized males to ensure estrus synchrony. Seventy-two hours following implant removal, embryos were recovered surgically from the oviducts of donors. Embryos were flushed from oviducts associated with ovulated ovaries through a cannula with sterile phosphate-buffered saline and were collected in a petri dish. The HindIII-BamHI fragment of WAP-LAtPA was injected into one of the two pronuclei from one-cell embryos or into a nucleus of one blastomere of two-cell embryos, at a concentration of 1μg/ml, using standard protocols. See, for example, page 7: line 17 - page 9, line 3, and page 11, lines 5-9 of the present application; Gordon et al. (1983) *Methods in Enzymology* 101:411; Overbeek et al. (1985). *Proc. Nat'l Acad. Sci* 82: 7815-7819;

and Gordon et al. (1980) *Proc. Nat'l Acad Sci* 77: 7380-7384. Embryos were surgically transferred into the oviducts of the recipient females or to the uteri following a 72 hour culture period, and allowed to undergo the remainder of gestation. As described above with respect to transgenic mice, tissue samples from newborn goats were analyzed by Southern blotting in order to detect transgenics.

14. Female goats identified as transgenic were mated to non-transgenic males. Commencing after parturition, transgenic mothers were milked manually over a period of 240 days. LAtPA concentrations (amidolytic activity) in milk samples were determined by an indirect method using the plasmin substrate Val-Leu-Lys-p-nitroanilide (S-2251, Helena Labs, Inc.). LAtPA concentration was also estimated using the IMUBIND tPA ELISA assay kit (American Diagnostics, Chicago IL) adapted to determine LAtPA in goat's milk. To illustrate the results of such analysis, one transgenic goat, which produced an average daily yield of milk in the range of 3-4 liters, expressed LAtPA at approximately 3 μg/ml during the peak lactation period (1 to 140 days) with an increase in concentration (6.0 μg/ml) toward the end of the lactation period (141 to 240 days).

15. It was demonstrated that targeting transgenes that coded for biologically active proteins to the mammary gland of a mammal is a feasible means of expressing and secreting foreign proteins into the milk of a transgenic animal. Concerns that foreign proteins produced in the mammary gland might not be secreted, accurately processed, or be sufficiently stable in milk were reduced by these results. The advantages of producing such recombinant proteins in this manner include the fact that milk is well characterized biochemically and that many of the genes encoding key milk proteins have been cloned. In addition, many milk-specific genes are expressed in the lactating mammary gland at high levels under hormonal control and in a tissue-specific manner. Thus, I believe that the presently claimed methods for producing a recombinant protein represent the means which makes it possible to target foreign gene expression to the

lactating mammary epithelium, and direct the secretion of the gene product into the milk of a transgenic mammal.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that such willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Katherine Gordon

Dated: October 4 1993

Signature: